IFW 2

AMENDMENT

AMENDMENTS TO THE SPECIFICATION

Please replace the paragraph located on page 19, lines 27, extending to page 20, line 5, with the following replacement paragraph:

-- The analysis step (ii) preferably comprises PCR using at least one pair of suitable primers. In the case where the gene is the pig αMSHR gene, the at least one pair of suitable primers is:

αMSHR Forward Primer 1: (5'-TGT AAA ACG ACG GCC AGT RGT GCC TGG AGG TGT-3') (SEQ ID NO:1)

αMSHR Reverse Primer 5: (5'-CGC CCA GAT GGC CGC GAT GGA CCG-3') (SEQ ID NO:2); or

αMSHR Forward Primer 2: (5'-CGG CCA TCT GGG CGG GCA GCG TGC-3') (SEQ ID NO:3)

αMSHR Reverse Primer 2: (5'-GGA AGG CGT AGA TGA GGG GGT CCA-3') (SEQ ID NO:4); or

αMSHR Forward Primer 3: (5'-GCA CAT CGC CCG GCT CCA CAA GAC-3') (SEQ ID NO:5)

αMSHR Reverse Primer 3: (5'-GGG GCA GAG GAC GAC GAG GGA GAG-3') (SEQ ID NO:6).--

Please replace the paragraph located on page 24, lines 3-9, with the following replacement paragraph:

-- In a preferred method PCR is carried out using primers that amplify a region of the KIT gene containing nucleotide 2678. An example of a suitable pair of primers is:
forward primer
LA93 5'-GAGCAGCCCCTACCCCGGAATGCCAGTTGA-3' (SEQ ID NO:7)

and the reverse primer

KIT56 5'-CTTTAAAACAGAACATAAAAGCGGAAACATCATGCGAAGG-3' (SEQ ID NO:8).--

Please replace the paragraph located on page 28, lines 21, extending to page 29, line 17, with the following replacement paragraph:

-- The αMSHR gene was amplified for sequence analysis using three primer pairs.
Primers MSHR Forward Primer 1: (5'-TGT AAA ACG ACG GCC AGT RGT GCC TGG AGG TGT CCA T-3') (SEQ ID NO:9); and
MSHR Forward Primer 5: (5'-CGC CCA GAT GGC CGC GAT GGA CCG-3') (SEQ ID NO:10)
amplify a 428 bp fragment from the 5' half of the gene.
Primers MSHR Forward Primer 2: (5'-CGG CCA TCT GGG CGG GCA GCG TGC-3') (SEQ ID NO:3);
and αMSHR Reverse Primer 2: (5'-GGA AGG CGT AGA TGA GGG GGT CCA-3') (SEQ ID NO:4)
amplify a 405 bp fragment the 3' half of the gene.
As these two fragments are non-overlapping a third primer pair
αMSHR Forward Primer 4 (5'-TGC GCT ACC ACA GCA TCG TGA CCC TGC-3') (SEQ ID NO:11); and
αMSHR Reverse Primer 4 (5'-GTA GTA GGC GAT GAA GAG CGT GCT-3') (SEQ ID NO:12)
were used to amplify a 98 bp fragment which spans the 50 bp gap. PCR was carried out on a DNA thermal cycler (Perkin Elmer 9600) in a total volume of 20 µl containing 25 ng genomic DNA, 1.0 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 200 (M dNTPs, 0.5 U AmpliTaq Gold (Perkin Elmer) and 10 pmol of both forward and reverse primer. To activate AmpliTaq Gold, initial heat denaturation was carried out at 94 degrees C for 10 minutes followed by 32 cycles each consisting of 45 sec at 94 degrees C, 45 sec at 53 degrees C and 45 sec at 72 degrees

C. The final extension lasted for 7 min at 72 degrees C. PCR products were cloned into vector pUC18 using the SureClone ligation kit (Pharmacia).--

Please replace the paragraph located on page 31, lines 3-14, with the following replacement paragraph:

-- Reactions were set up in a 20µl reaction volume in thin walled 0.25ml tubes (Perkin Elmer) with the following components:

20µl reaction volume:

2µl template DNA

1.5 mM $MgCl_2$

200µM each dNTP,

3pM each of forward and reverse primers

0.5 U AmpliTaq Gold (Perkin Elmer)

MSHR Forward primer 3 sequence: 5' GCA CAT CGC CCG GCT CCA CAA GAC 3' (SEQ ID NO:5)

MSHR Reverse primer 3 sequence: 5' GGG GCA GAG GAC GAC GAG GGA GAG 3' (SEQ ID NO:6).--

Please replace the paragraph located on page 37, lines 1-10, with the following replacement paragraph:

-- Reactions were set up in a 20µl reaction volume in thin walled 0.25ml tubes (Perkin Elmer) with the following components:

10µl reaction volume:

2µl template DNA

2.5 mM $MgCl_2$

200µM each dNTP,

5pmol each of forward and reverse primers

0.5 U AmpliTaq Gold (Perkin Elmer)

Forward primer sequence: 5' CTG CCT GGC CGT GTC GGA CCT G **3'** (SEQ ID NO:13)

Reverse primer sequence: 5' CTG TGG TAG CGC AGC GCG TAG AAG 3' (SEQ ID NO14).--

Please replace the paragraph located on page 39, lines 4-17, with the following replacement paragraph:

-- DNA was prepared from cattle muscle samples as described in example 4. PCR was then carried out in a 100µl reaction using the primer pair:

5'-TGAGGTAGGAGAGTTTTGGG-3' (SEQ ID NO:15) and

5'-TCGAAATTGAGGGGAAGACC-3' (SEQ ID NO:16)

as described in Kambadur *et al. Genome Research* 7: 910-915 (1997) at a concentration of 500nM with other reaction components being 2.5mM $MgCl_2$, 200µM dNTPs, 50mM KCl, 10mM Tris-HCl pH 8.3, 5 units AmpliTaq Gold (Perkin Elmer). 1µl of bovine genomic DNA was used as template. Denaturation was carried out for 12 min at 94°C followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72° 1.5 min followed by 5 min at 72°C. Following PCR 2.0µl of loading dye (44.5mM Tris pH 8.0, 44.5mM boric acid, 0.5mM EDTA, 50%w/v glycerol, 0.02% w/v Orange G) was added to 10µl of product and analysis carried out by electrophoresis on a 2% agarose gel prepared in 0.5x TBE buffer (44.5mM Tris pH 8.0, 44.5mM boric acid, 0.5mM EDTA) for 1 hour at 100V.--

Please replace the paragraph located on page 40, lines 11-26, with the following replacement paragraph:

-- First-strand cDNA synthesis was accomplished using the First-Strand cDNA Synthesis kit (Pharmacia Biotech) so that ~100 ng mRNA was randomly primed by 0.1 µg pd(N6) in a total

volume of 15 µl. Two µl of the completed first cDNA strand reaction was then directly used per 12 µl PCR reaction by adding 10 µl PCR mix containing 10 pmol each of the mouse/human derived primers KIT1F and KIT7R (5'-TCR TAC ATA GAA AGA GAY GTG ACT C (SEQ ID NO:17) and 5'-AGC CTT CCT TGA TCA TCT TGT AG (SEQ ID NO:18), respectively; Moller et al. 1996, *supra*), 1.2 µl 10 x PCR-buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl) and 0.5 U of AmpliTaq polymerase (Perkin-Elmer) incubated with an equal amount Taqstart antibody (Clontech) at 25 °C for 5 min to achieve a hot start PCR. The reaction was covered with 20 µl mineral oil and thermocycled in a Hybaid Touchdown machine (Hybaid) with 40 cycles at 94°C for 1 min, 55-48 °C (touchdown one degree per cycle the first seven cycles and then 48°C in the remaining cycles) for 1 min and 72°C for 1 min. After PCR 2µl loading dye was added to each sample which were then loaded on 4% agarose gel (Nusieve/Seakem 3:1, FMC Bioproducts) and electrophoresed with 100V for 80 min. Products were visualised by ethidium bromide staining and UV-illumination.--

Please replace the paragraph located on page 42, lines 2-12, with the following replacement paragraph:

--A 175 bp region including the boundary between exon17 and intron17 of the *KIT* gene was amplified for sequence analysis using forward primer KIT21 (5' - GTA TTC ACA GAG ACT TGG CGG C -3') (SEQ ID NO:19) and reverse primer KIT35 (5' - AAA CCT GCA AGG AAA ATC CTT CAC GG - 3') (SEQ ID NO:20). PCR was carried out on a DNA thermal cycler (Perkin Elmer 9600) in a total volume of 20 µl containing 25 ng genomic DNA, 1.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 200 µM dNTPs, 0.5 U AmpliTaq Gold (Perkin Elmer) and 10 pmol of both KIT21 and KIT35 primer. To activate AmpliTaq Gold, initial heat denaturation was carried out at 94°C for 10 minutes followed by 32 cycles each consisting of 45 sec at 94°C, 45 sec at 55°C and 45 sec at 72°C. The final extension lasted for 7 min at 72°C. PCR products were cloned into vector pUC18 using the SureClone ligation kit (Pharmacia Biotech).--

Please replace the paragraph located on page 49, lines 16, extending to page 50, line 1, with the following replacement paragraph:

--*KIT* primers

Forward GAATATTGTTGCTATGGTGATCTCC *KIT*1-FOR (SEQ ID NO:21)

Reverse CCGCTTCTGCGTGATCTTCCTG *KIT*1-REV (SEQ ID NO:22)

CRC primers

Forward CTGGATGTCCTGTGTTCCCTGT CRC-FORWARD (SEQ ID NO:23)

Reverse AGGTTTGTCTGCAGCAGAAGCTC CRC-REVERSE (SEQ ID NO:24)

The reverse *KIT* primer and the forward *CRC* primer are labelled with the ABI fluorescent dye FAM at the 5' end.--

Please replace the paragraph located on page 53, lines 10-13, with the following replacement paragraph:

-- *KIT* primers

Forward GAAAGTGA(C/T)GTCTGGTCCTAT(C/G)GGAT *KIT*DEL2-FOR (SEQ ID NO:25)

Reverse AGCCTTCCTTGATCATCTTGTAG *KIT*DEL2-REV (SEQ ID NO:26)--

Please replace the paragraph located on page 56, lines 21-24, with the following replacement paragraph:

-- Primers

forward TGTGGGAGCTCTTCTCTTTAGG *KIT*DEL1-FOR (SEQ ID NO:27)

reverse CCAGCAGGACAATGGGAACATCT *KIT*DEL1-REV (SEQ ID NO:28)
The reverse primer was labelled with the ABI fluorescent dye FAM at the 5' end.--

Please replace the paragraph located on page 58, lines 15, extending to page 59, line 5, with the following replacement paragraph:

--mRNA was isolated from peripheral blood leukocytes from white (Landrace/Large White) and coloured (Hampshire) pigs using the Message Maker mRNA isolation system (Gibco BRL) with one mRNA selection from total RNA. 100ng poly(A)$^+$ mRNA was reverse-transcribed with random primers (First-Strand cDNA Synthesis kit, Pharmacia Biotech) and the product was used at a 1:10 dilution for RT-PCR using the proof-reading Advantage KlenTaq Polymerase (Clontech) according to the manufacturer's recommendation. The following primers were used to amplify almost the entire coding sequence and some of the 5' untranslated region: KIT40 (5'-GGC TCT GGG GGC TCG GCT TTG C) (SEQ ID NO:29) corresponding to the 5'untranslated region and KIT22S (5'- TCA GAC ATC TTC GTG GAC AAG CAG AGG) (SEQ ID NO:30) corresponding to exon 21; both primers had been designed using consensus sequence of the human and mouse *KIT* sequences in the GENBANK database. The RT-PCR products were gel purified and cloned using the pGEM-T vector system (Promega). Plasmid clones were sequenced using a set of internal primers and the ABI Prism™ dRhodamine Terminator Cycle Sequencing Kit (PE Applied Biosystems). Two subclones representing each type of *KIT* sequence were initially sequenced and in those cases where a discrepancy was observed (possibly due to PCR errors) additional clones were sequenced over those particular nucleotide sites. RT-PCR analysis of *KIT* exon 16-19 was carried out with the primers KIT1F (5'-TCR TAC ATA GAA AGA GAY GTG ACT C) (SEQ ID NO:31) and KIT7R (5'-AGC CTT CCT TGA TCA TCT TGT AG) (SEQ ID NO:32).--

Please replace the paragraph located on page 60 with the following replacement paragraph:

Breed	**Coat Colour**	**Assumed Genotype**	**Sequence variant**	**Splicing exon 14**	**Splicing exon 17**	**Exon 5 821**	**Exon 5 828**	**Exon 6 978**	**Exon 6 984**	**Exon 6 1008**	**Exon 9 1464**	**Exon 18 2502**	**Exon 19 2678**	**SEQ ID NO:**
Hampshire	coloured	i/i	KIT1*0101	normal	normal	AGG Arg	ACA Thr	AAC Asn	GGA Gly	GAG Glu	ACG Thr	CCT Pro	GCG Ala	33 34
Yorkshire / Landrace	white	I/I[1]	KIT1*0201	normal	normal	-	-	AAT Asn	GGC Gly	GAA Glu	ACA Thr	CCC Pro	GTG Val	35 36
			KIT1*0202	normal	normal	AAG Arg	ACG Thr	-	-	GAA Glu	ACA Thr	CCC Pro	GTG Val	37 38
			KIT2*0101	normal	skipped	-	-	AAT Asn	GGC Gly	GAA Glu	ACA Thr	CCC Pro	GTG Val	39 40
			KIT2 splice variant	skipped [2]	skipped	-	-	AAT Asn	GGC Gly	GAA Glu	ACA Thr	CCC [2]	GTG [2]	41 42

[1] Genotype I/I, I/I* or I*/I* inferred by the pig being a sow that got a 100 % white farrow following mating to a Hampshire (i/i) boar.

[2] The skipping of exon 14 (151 bp) cause a nonsense translation with termination at position 2161.--

Please replace the paragraph located on page 61, lines 5-13, with the following replacement paragraph:

--A 158bp fragemnt covering 99bp of the end of exon 19 and 59bp of the KIT gene was amplified using forward primer LA93 (5'-GAG CAG CCC CTA CCC CGG AAT GCC AGT TGA-3') (SEQ ID NO:7) and reverse primer KIT56 (5'-CTT TAA AAC AGA ACA TAA AAG CGG AAA CAT CAT GCG AAG G-3') (SEQ ID NO:8). PCR was carried out on a Perkin Elmer 9600 Thermal Cycler in a total volume of 20µl containing 25ng genomic DNA, 1.5mM $MgCl_2$, 50mM Kcl, 10mM Tris-HCl, pH 8.3, 200µM dNTPs, 0.5u AmpliTaq Gold (Perkin Elmer) and 10 pmol of both LA93 and KIT56 primer. To activate AmpliTaq Gold, initial heat denaturation was carried out at 94°C for 10 minutes followed by 32 cycles each consisting of 45 sec at 94°C, 45 sec at 55°C and 45 sec at 72°C.--

Please replace the paragraph located on page 63, lines 9-25, with the following replacement paragraph:

-- Allelic discrimination reactions were set up using the PE Applied Biosystems TaqMan™ system. 25µl reactions contained the primers E19FOR (5-GAGCAGCCCCTACCCCGGAATGCCAGTTGA-3') (SEQ ID NO:43) and E19REV (5-CTTTAAAACAGAACATAAAAGCGGAAACATCATGCGAAGG-3') (SEQ ID NO:44) at 300nM, 8% glycerol (w/v) 1X TaqMan™ buffer A (PE Applied Biosystems), 5mM $MgCl_2$, 200µM dATP, dGTP, dCTP and dUTP, 0.65 units AmpliTaq Gold™ (PE Applied Biosystems), 0.25 units AmpErase™ UNG (PE Applied Biosystems) and the TaqMan™ probes E19PC (5'-CATACATTTCCGCAGGTGCATGC-FAM) (SEQ ID NO:68) and E19PT (5'-TCATACATTTCCACAGGTGCATGC-TET) (SEQ ID NO:69) at a concentration of 100mM. 1µl of crude lysate DNA was used as template. PCR amplification was carried out using a PE9600 thermal cycler (PE Applied Biosystems) or a the ABI7700 Prism (PE Applied Biosystems) with a thermal cycling regime of 50°C for 2 min followed by 95°C for 10 min followed by 40 cycles of 95°C 15 sec, 62°C 1 min. 8 control samples of each homozygote genotype, 2678C and 2678T, and

8 no template controls where deionized water was substituted for template controls were used per 96 well plate. Allele identification based on these reactions was carried out using the allelic discrimination function of the ABI7700 Prism (PE Applied Biosystems).--

Please replace the paragraph located on page 64, lines 16-26, with the following replacement paragraph:

--A 158 bp fragment covering 99 bp of the 3' end of exon 19 and 59 bp of intron 19 of the KIT gene was amplified using the following primers:
forward LA93 (5' - GAGCAGCCCCTACCCCGGAATGCCAGTTGA -3') (SEQ ID NO:7); and reverse
KIT56 (5' -CTTTAAAACAGAACATAAAAGCGGAAACATCATGCGAAGG -3') (SEQ ID NO:8). PCR was carried out in a total volume of 20 µl containg 25 ng genomic DNA, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM tris-HCl, pH 8.3, 200 µM dNTPs, 0.5 U AmpliTaq Gold (Perkin Elmer) and 10 pmol of both LA93 and KIT56 primer. To activate Amplitaq Gold, initial heat denaturation was carried out at 94°C for 10 minutes followed by 32 cycles each consisting of 45 sec at 94°C, 45 sec at 55°C and 45 sec at 72°C.--

Please replace the paragraph located on page 66, line 23, extending to page 67, line 6, with the following replacement paragraph:

--The entire coding region of the *αMSHR* gene was amplified from genomic DNA using primers EPIG10 and EPIG16. These primers have sequence:
EPIG10 5' - GGT CTA GAT CAC CAG GAG CAC TGC AGCACC -3' (SEQ ID NO:45)
EPIG16 5' - GGG AAG CTT GAC CCC CGA GAG CGA CGC GCC-3' (SEQ ID NO:46)
PCR was carried out on a DNA thermal cycler (Perkin Elmer 9600) in a total volume of 20 µl containing 25 ng genomic DNA, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM TriHC1, pH 8.3, 200 µM dNTPs, 5.0 % DMSO (dimethyl sulfoxide), 0.5 U AmpliTaq Gold (Perkin Elmer) and

10 pmol of both EPIG10 and EPIG16. To activate AmpliTaq Gold, initial heat denaturation was carried out at 96°C for 10 minutes followed by 32 cycles each consisting of 45 sec at 94°C, 45 sec at 55°C and 45 sec at 72°C. The final extension lasted for 7 min at 72°C.--

Please replace the paragraph located on page 68, lines 11-33, with the following replacement paragraph:

--A 2bp insertion was identified in the aMSHR gene of pigs of the Pietrain breed which carry the E^p allele between nucleotide positions equivalent to 66 and 67 in the Wild Boar αMSHR sequence. This results in a shift in the translation frame and creates a TGA stop codon at nucleotide positions equivalent to 161 to 163 in the Wild Boar αMSHR sequence. The 5' portion of the *αMSHR* coding sequence compared between three breeds is shown below. This comparison illustrates the two base pair insertion present within the alleles carried by the Pietrain animal when compared with either the Hampshire or Wild Boar alleles.. The ATG start codon is highlighted in bold, the 3' end of primer EPIG16 is shown in italics and bases in common with the Pietrain sequence are marked with a dash. Missing bases are marked with :.

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Pietrain   CGACGCGCCC  TCCCTGCTCC  CTGGCGGGAC  GATGCCTGTG CTTGGCCCGG
Meishan    ----------  ----------  ----------  ---------- ----------
Wild Boar  ----------  ----------  ----------  ---------- ----------

Pietrain   AGAGGAGGCT  GCTGGCTTCC  CTCAGCTCCG  CGCCCCCAGC CGCCCCCCCC
Meishan    ----------  ----------  ----------  --------:: ----------
Wild Boar  ----------  ----------  ----------  --------:: ----------

Pietrain   GCCTCGGGCT  GGCCGCCAAC  CAGACCAACC  AGACGGGCCC CCAGTGCCTG
Meishan    ----------  ----------  ----------  ---------- ----------
Wild Boar  ----------  ----------  ----------  ---------- ----------

Pietrain   GAGGTGTCCA  TT  (SEQ ID NO:47)
Meishan    ----------  --  (SEQ ID NO:48)
WildBoar   ----------  --  (SEQ ID NO:48)
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These results are also incorporated into figure 1a.--

Please replace the paragraph located on page 69, lines 4-15, with the following replacement paragraph:

-- PCR was conducted with forward primer EPIG16 (see above) and reverse primer MC1R121A exactly as described above. The reverse primer was labeled with ABI dye Hex and has sequence:
MC1R121A 5' - Hex- GGA CTC CAT GGA GCC GCA GAT GAG CAC GGT 3' (SEQ ID NO: 49).
Following PCR cycling, 0.2 µl of the reaction volume was mixed with 2.5 µl of deionised formamide, 0.5 µl of GS500 DNA standard (ABI) and 0.4 µl blue dextran solution before being heated to 90°C for 2 minutes and rapidly cooled on ice. 1 µl of this mix was then loaded onto a 377 ABI Prism sequencer and the DNA fragments separated on a 6% polyacrylamide gel in 1 X TBE buffer for 2 hours at 700 V, 40 mA, 32 W. The length of the resulting PCR products were determined using the GeneScan software (ABI).--

Please replace the paragraph located on page 70, lines 13-23, with the following replacement paragraph:

--A 454 bp product containing 38 coding nucleotides from the 3' portion of the molecule and 416 bp of 3' untranslated region (not including primer binding sites) was amplified using primers EPIG13 and EPIG14. These primers have sequence:
EPIG13 5'-GCA AGA CCC TCC AGG AGG TG-3' (SEQ ID NO:50)
EPIG14 5'-CAC TGA GCC GTA GAA GAG AG- 3' (SEQ ID NO:51)
PCR was carried out on a DNA thermal cycler (Perkin Elmer 9600) in a total volume of 20 µl containing 25 ng genomic DNA, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH8.3, 200 µM dNTPs, 0.5 U AmpliTaq Gold (Perkin Elmer) and 10 pmol of both EPIG13 and EPIG14. To activate AmpliTaq Gold, initial heat denaturation was carried out at 96°C for 10 minutes

followed by 32 cycles each consisting of 45 sec at 94° C, 45 sec at 55°C and 45 sec at 72°C. The final extension lasted for 7 min at 72°C.--